

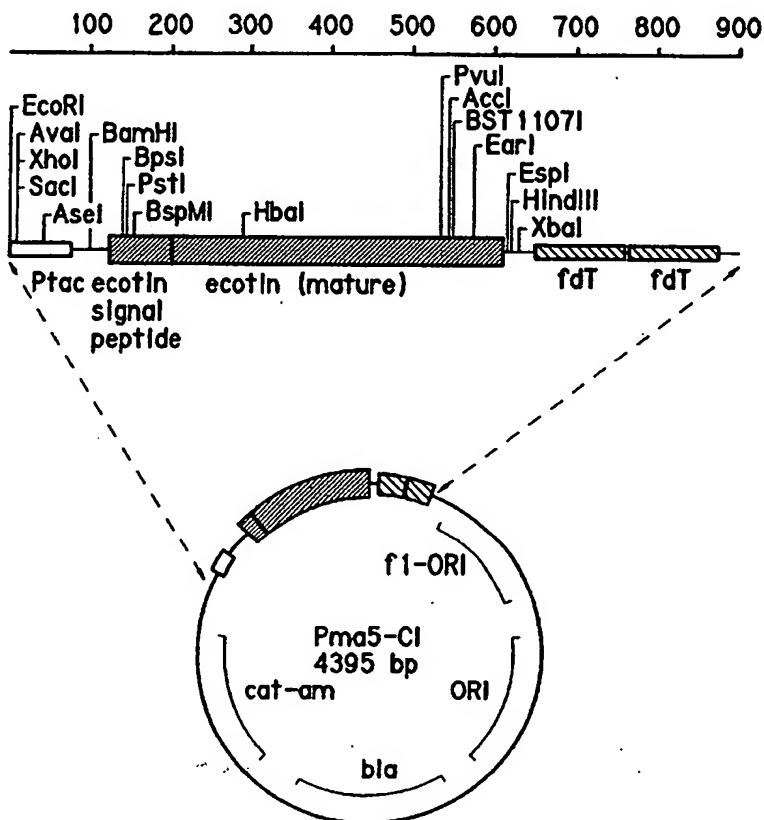


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<b>(21) International Application Number:</b> PCT/US93/12003 <b>(22) International Filing Date:</b> 9 December 1993 (09.12.93)  <b>(30) Priority Data:</b> 07/988,984 11 December 1992 (11.12.92) US  <b>(71) Applicant:</b> CORVAS INTERNATIONAL, INC. [US/US]; 3030 Science Park Road, San Diego, CA 92121 (US).  <b>(72) Inventors:</b> LAUWEREYS, Marc, Jozef; Wilgenstraat 2, B-Haaltert (BE). LAMBIER, Anne-Marie, Virgine, Renée; Fonteinstraat 12, B-Oud-Heverlee (BE).  <b>(74) Agents:</b> WARBURG, Richard, J. et al.; Lyon & Lyon, 34th floor, 611 West Sixth Street, Los Angeles, CA 90017 (US).		<b>(81) Designated States:</b> CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>

**(54) Title:** ECOTIN AS A FACTOR Xa, XIa, AND XIIa INHIBITOR**(57) Abstract**

Pharmaceutical composition containing Ecotin in a therapeutically effective amount for inhibition of blood coagulation.



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## ECOTIN AS A FACTOR Xa, XIa, AND XIIa INHIBITOR

Field of the Invention

The present invention relates to pharmaceutical compositions including Ecotin, as well as methods for use of Ecotin as a factor Xa, XIa and XIIa inhibitor.

Background of the Invention

Ecotin is a known serine protease inhibitor. The gene encoding this protein has been cloned, and the enzyme's properties characterized. The following is a brief summary of the known features of Ecotin:

Chung et al., 258 J. Biol. Chem. 11032, 1983, describe purification of a periplasmic protein, which they termed Ecotin, that is a potent inhibitor of pancreatic proteases, such as chymotrypsin, pancreatic elastase, rat mast cell chymase, and human serosal urokinase. The inhibitor does not inhibit human pulmonary tryptase, kallikrein, papain, pepsin, *Staphylococcus aureus* V8 protease, subtilisin, and thermolysin. Further, it does not inhibit any of the eight soluble endoproteases which are isolated from *E. coli* (including the proteases Do, Re, Mi, Fa, So, La, Ci, and Pi), nor the chymotrypsin-like (protease I) and trypsin-like (protease II) esterases in *E. coli*.

Palmer and St. John, 169 J. Bacteriology 1474, 1987, describe three membrane-associated serine proteases

that are insensitive to inhibition by Ecotin, despite those proteolytic activities being present in *E. coli*.

McGrath et al., 266 J. Biol. Chem. 6620, 1991, describe Ecotin as an unusual serine protease inhibitor  
5 since it is able to inhibit chymotrypsin, trypsin and elastase.

McGrath et al., 222 J. Molecular Biol. 139, 1991, describes expression of Ecotin in the *E. coli* periplasm, using the endogenous signal peptide and the heterologous  
10 tac promoter.

Lee et al., 287 FEBS Letters 53, 1991, describe a nucleotide sequence of 876 bases in the *E. coli* chromosome that encodes Ecotin. They proposed that the coding sequence for Ecotin is 486 nucleotides long, and encodes a  
15 protein consisting of 162 amino acids.

#### Summary of the Invention

Applicant has surprisingly discovered that Ecotin is a potent inhibitor of factor Xa, XIa, and XIIa. Applicant proposes that Ecotin can be used in medical  
20 research as an in vitro diagnostic reagent to inhibit the activity of factor Xa, XIa and XIIa. Applicant further proposes that Ecotin can be used as a therapeutic or pharmacological agent by providing Ecotin in a pharmaceutically acceptable buffer in a therapeutic amount  
25 within a mammal having an elevated factor Xa activity and/or elevated factor XIa and XIIa activity.

Thus, in a first aspect, the invention features a pharmaceutical composition containing Ecotin in a therapeutically effective amount.

30 By "Ecotin" is meant that compound or inhibitor described in those publications noted above, and more fully characterized below, and derivatives thereof. Those in the art will recognize that derivatives of Ecotin can be

readily formed which have the inhibitory activity of Ecotin for factor Xa, XIa and XIIa but may have slightly altered amino acid sequences. For example, those of ordinary skill in the art can perform routine shotgun mutagenesis, or  
5 specific point mutagenesis, using known techniques to create random or defined mutants of Ecotin. Such mutants can be expressed to produce derivatives of Ecotin, and then readily screened for mutants which retain the desired factor Xa, XIa and XIIa inhibitory activities, as described  
10 below. Thus, the invention features not only use of Ecotin, but also analogs, homologs, or other equivalent derivatives thereof.

By "pharmaceutical composition" is meant a composition which includes a suitable vehicle for  
15 introduction of Ecotin into a human or other mammal in need of an inhibitor of factor Xa, XIa and XIIa.

The present invention also encompasses pharmaceutical compositions prepared for storage and subsequent administration, which have a pharmaceutically  
20 effective amount of Ecotin disclosed above in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co.  
25 (A.R. Gennaro edit. 1985). Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. For example, sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid may be added as preservatives. Id. at 1449. In addition,  
30 antioxidants and suspending agents may be used. Id.

The compositions of the present invention may be formulated and used as tablets, capsules or elixirs for oral administration; suppositories for rectal

administration; sterile solutions, suspensions for injectable administration; and the like. Injectables, which are particularly suited for treatment of blood coagulation, can be prepared in conventional forms, either  
5 as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Suitable excipients are, for example, water, saline, dextrose, mannitol, lactose, lecithin, albumin, sodium glutamate, cysteine hydrochloride, and the like. In  
10 addition, if desired, the injectable pharmaceutical compositions may contain minor amounts of nontoxic auxiliary substances, such as wetting agents, pH buffering agents, and the like. If desired, absorption enhancing preparations (e.g., liposomes) may be utilized.

15         The present invention also features a method for preventing or treating a condition in a mammal characterized by an elevated factor Xa level, e.g., abnormal thrombosis or blood coagulation. The pharmaceutically effective amount of the composition  
20 required as a dose will depend on the route of administration, the type of animal being treated, and the physical characteristics of the specific animal under consideration. The dose can be tailored to achieve optimal efficacy, but will depend on such factors as weight, diet,  
25 concurrent medication and other factors which those skilled in the medical arts will recognize.

          In practicing the methods of the invention, the compositions can be used alone or in combination with other effective agents, e.g., in combination with other  
30 therapeutic or diagnostic agents. These compounds can be utilized *in vivo*, ordinarily in a mammal, preferably in a human, or *in vitro*. In employing them *in vivo*, the compounds or compositions can be administered to the mammal

in a variety of ways, including parenterally, subcutaneously, intramuscularly, colonically, rectally, nasally or intraperitoneally, employing a variety of dosage forms. Most preferably, the material is administered intravenously for treatment of a blood coagulation disorder.

As will be readily apparent to one skilled in the art, the useful *in vivo* dosage to be administered and the particular mode of administration will vary depending upon the age, weight and mammalian species treated, the particular Ecotin derivative employed, and the specific use for which these derivatives are employed. The determination of effective dosage levels, that is the dosage levels necessary to achieve the desired result, will be within the ambit of one skilled in the art. Typically, applications of compound are commenced at lower dosage levels, with dosage level being increased until the desired effect is achieved.

The dosage for Ecotin of the present invention can range broadly depending upon the desired affects and the therapeutic indication. Typically, dosages will be between about 0.01  $\mu\text{g/kg}$  and 100  $\text{mg/kg}$  body weight, preferably between about 0.01  $\mu\text{g/kg}$  and 10  $\text{mg/kg}$  body weight. Administration is preferably parenteral, such as intravenous on a daily basis.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

#### Description of the Preferred Embodiments

The drawings will first briefly be described.

#### Drawings

Fig. 1A shows nucleotide sequences used in the construction of pMa5-CI. Specifically, it shows the

relevant part of the recipient vector pMa5-19. The EcoRI/XbaI fragment is present in the multi-cloning site of pMa/c5-8 (Stanssens et al., 17 Nucl. Acids Res. 4441, 1989). The -35 and -10 box of the  $P_{lac}$  promoter, the Shine-Dalgarno (SD) sequence and secretion signal derived from the *phoA* gene as well as some restriction sites are indicated. Fig. 1B shows the sequence of the Ecotin-encoding PCR-product obtained with the primers Pst390 and Pst391. The BamHI and HindIII sites used for the directional cloning of the PCR-product in the recipient vector pMa5-19 are shown. The Ecotin secretion signal is underlined.

Fig. 2 is a diagrammatic representation of the pMa5-CI vector. The map of the pMa5-CI vector contains the following relevant features: (i) a ColE1 type origin of replication (ORI); (ii) the intercistronic region of filamentous phage f1 including the origin of replication (f1-ORI); (iii) the beta-lactamase gene which confers resistance to ampicillin (*bla*); (iv) the chloramphenicol acetyl transferase gene containing a single point mutation which creates an amber translational stop codon (*cat-am*); (v) the Ecotin expression module consisting of the IPTG-inducible *tac*-promoter, the Ecotin gene with its indigenous secretion signal and two copies of the central transcription terminator of phage fd (*fdT*).

Fig. 3 is a reproduction of an SDS polyacrylamide gel (15% acrylamide) Coomassie Blue stained, showing molecular weight markers in lane 1, factor Xa inhibitory protein from periplasmic fraction in lane 2, and factor Xa inhibitory protein from total lysate in lane 3.

Fig. 4 is a graph showing titration of a 1/20 dilution (circles) of a 2% TCA supernatant of a crude



*E. coli* lysate with factor Xa. Rectangles show the initial rates in absence of inhibitor.

#### Ecotin

Applicant surprisingly discovered a factor Xa, XIa, and XIIa inhibitory activity in *E. coli* lysates. This activity was later identified as Ecotin. The following is an example of the identification of Ecotin as a potent factor Xa, XIa, and XIIa inhibitor, and the processes and procedures by which Ecotin can be isolated, purified and then produced by recombinant DNA techniques. Those of ordinary skill in the art will recognize that these examples are not limiting in the invention, and that they can readily devise equivalent procedures by which Ecotin can be purified or expressed, and then used in factor Xa, XIa, and XIIa assays.

As discussed above, the assays described below can be used to identify derivatives of Ecotin in which a few amino acids are altered, added or deleted, but which retain the Ecotin factor Xa inhibitory activity. Indeed, those experiments can be used to more carefully define the active portion of Ecotin responsible for inhibition of factor Xa, XIa, and XIIa activity. Thus, the invention also features (and the term "Ecotin" includes in its definition) use of smaller derivatives of Ecotin in which one or more amino acid sequences are deleted from the native Ecotin protein.

#### Examples

During routine experiments, WK6 *E. coli* cells harboring a vector, thought to contain a gene coding for a putative factor Xa, XIa, and XIIa inhibitor, were cultured and, after lysis, were found to contain a potent inhibitor for trypsin and factors Xa, XIa and XIIa. The concentration of the inhibitor was estimated by titration

of a fixed amount of crude lysate with increasing factor Xa concentrations. The same experiment allows estimation of the inhibition constant of the inhibitor. In a particular experiment, the inhibitor concentration was about 12 nM, and the inhibition constant for factor Xa was in the range of 10 pM.

Subsequently, the inhibitory compound was isolated and found not to be the protein encoded by the vector, but rather a form of the endogenous *E. coli* trypsin inhibitor known as Ecotin. The presence of a potent factor Xa inhibitor could also be demonstrated in *E. coli* cells, carrying a different vector. The methods used to measure inhibitor properties, and to purify the inhibitor and characterize the protein are given below.

Example 1: Cell Growth and Lysis

Transformed *E. coli* (WK6, containing vectors thought to express a factor Xa, XIa, and XIIa inhibitor) were grown in 250 ml baffled Erlenmeyer flasks at 37°C in the presence of ampicillin. The cells were induced after 3 hours by addition of 0.1 mM IPTG and grown overnight.

In one particular procedure the total *E. coli* soluble fraction was used for the isolation, using the protocol described by Marks et al., 261 J. Biol. Chem. 7115, 1986.

Specifically, about 1 g of wet cell paste was suspended in 1.5 ml 40 mM TRIS buffer pH 8 containing 20% sucrose and 5 mM EDTA. 2.5 mg lysozyme was added, followed by 1.15 ml of 0.1% Triton X-100 and 0.3 ml NaCl (5 M). After 15 minutes at room temperature, 2.5 ml of 200 mM TEA buffer pH 7.8 was added followed by 0.15 ml CaCl<sub>2</sub> (1 M) and 0.1 ml MgCl<sub>2</sub> (1 M) and 10 µg of DNaseI. The suspension was stirred for 20 minutes at 25°C.

The majority of the protein in the suspension was precipitated by addition of trichloroacetic acid (TCA) to a final concentration of 2%, and aggregates removed by centrifugation. The TCA supernatant was neutralized by the addition of NaOH for further purification.

Example 2: Titration of Crude E. coli Lysate with Factor Xa

Two percent TCA supernatant was diluted 1/20 and 1/40 in TBSA (100 mM TRIS, 140 mM NaCl pH 7.4, 0.1% BSA), incubated with increasing amounts of factor Xa (0.05 - 4 nM) during 2 hours at room temperature (about 20°C) in microtiter plate wells. 0.25 mM factor Xa substrate was added (N- $\alpha$ -Cbo-D-Arg-Gly-Arg-pNa.2HCl) and the initial rate was determined at 405 nM during 40 minutes. The initial rate ( $v_i$ ) was plotted versus the factor Xa concentration ([E]) and fitted with the following equation to give an estimate of the inhibitor concentration ([I]) and the inhibition constant ( $K_i$ ).

$$v_i = Spa * \{([E] - [I] - K_i) + \sqrt{([I] + K_i - [E])^2 + 4 * K_i * [E]}\} / 2$$

Where Spa is the specific activity of factor Xa in the conditions of the assay.

The results of one such experiment are shown in Fig. 4. These results demonstrate a factor Xa inhibitory activity in the TCA supernatant.

Example 3: Purification of Factor Xa Inhibitor from TCA Supernatant

Purification of the inhibitor was achieved by affinity chromatography on Trypsin-Sepharose, equilibrated in 100 mM TEA pH 7.8, 300 mM NaCl, washed with 5 column volumes of 100 mM TEA pH 7.8, 300 mM NaCl and further washed with 5 column volumes of 10 mM TEA pH 7.8, 50 mM NaCl. The adsorbed material was eluted with 20 mM HCl, 50 mM NaCl pH 1.8. The eluted material was further

fractionated by reversed phase chromatography on Vydac C18. The column was equilibrated in 0.1% TFA/water solvent, and developed with a linear gradient of increasing acetonitrile from 0 to 50% at a flow rate of 1 ml/min. The fractions  
5 containing the inhibitory activity against factor Xa and Trypsin were subjected to SDS-PAGE electrophoresis (see Fig. 3, lane 3) and N-terminal sequence determination (see Example 5).

10 Example 4: Purification of Factor Xa Inhibitor from the  
E. coli Periplasmic Fraction

The inhibitor was also isolated from the periplasmic fraction of WK6 cells containing a vector thought to encode an inhibitor. Specifically, cells harvested from 0.6 L culture were resuspended in 72 ml 10  
15 mM Tris pH 7.5, 20% sucrose, 10 mM EDTA and left for 10 minutes at room temperature before centrifugation. The pellet was subsequently resuspended in 70 ml water. After 10 minutes the supernatant was collected by centrifugation and 4 ml NaCl (5 M) and 1.4 ml TEA pH 7.8 was added. After  
20 centrifugation the purification was achieved by affinity chromatography on Trypsin-Sepharose, equilibrated in 100 mM TEA pH 7.8, 300 mM NaCl, washed with 5 column volumes of 100 mM TEA pH 7.8, 300 mM NaCl and further washed with 5 column volumes of 10 mM TEA pH 7.8, 50 mM NaCl. The  
25 adsorbed material was eluted with 20 mM HCl, 50 mM NaCl pH 1.8. The eluted material was further fractionated by reversed phase chromatography on Vydac C4. The fraction containing the inhibitory activity for factor Xa and Trypsin was subjected to SDS-PAGE and had the same mobility  
30 on 15% SDS-PAGE as the protein purified from the total cell lysate (see Fig. 3, lane 2).

Example 5: Molecular Weight and N-terminal Sequence  
Determination

The inhibitor isolated from *E. coli* cells as described in Example 3, was subjected to automated gas  
5 phase Edman degradation for 15 cycles on an Applied Biosystems Sequencer. The deduced N-terminal amino acid sequence Ala-Thr-Thr-Ser-Ala-Trp-Ala-Ala-Glu-Ser-Val-Gln-Pro-Leu-Glu- is in agreement with the published sequence of the *E. coli* Trypsin inhibitor known as Ecotin. The first  
10 seven amino acids (underlined) of the purified material are part of the signal peptide, as deduced from the DNA sequence. McGrath et al., 266 J. Biol. Chem. 6620, 1991.

Example 6: Cloning of the Ecotin Gene on the pMa5-19  
Vector

15 The Ecotin gene was isolated from total DNA of *E. coli* strain WK6 by PCR-technology, and subsequently introduced in the vector pMa5-19 for expression purposes. For the preparation of total *E. coli* DNA, the cell-pellet of a 40 ml culture was resuspended in 15 ml TE (50 mM Tris  
20 pH 8.0, 20 mM EDTA). 5 ml of a proteinase K solution (2.5 mg/ml) was added and the mixture incubated for 2 hours at 37°C. After mixing with 5 ml sarcosyl (5% in TE) and incubation for another hour at 37°C (until clear), the mixture was extracted twice with 20 ml phenol and three  
25 times with ether. DNA was precipitated by the addition of 1/10 volume 5 M NaClO<sub>4</sub> and 1 volume isopropanol and collected by centrifugation (10,000 rpm, 10 minutes). The pellet was washed with 95% EtOH and resuspended in 4 ml TE. The quality of the material was checked by agarose-gel  
30 electrophoresis and the concentration determined spectrophotometrically.

The Ecotin gene, including the presumed secretion signal and ribosome binding site, was amplified using the following oligonucleotide-primers:

Pst 390: 5'-GCGACATCAT.CGGTGGGATC.CTATAAATGA.AGA

5

BamHI

Pst 391: 5'-GCACTTCACG.GAAGCTTAGC.GAACTACCGC

HindIII

Note that Pst390 and Pst391 contain a BamHI and HindIII restriction site, respectively, allowing the directional  
10 cloning of the obtained PCR product in the chosen expression vector (see below). For PCR-amplification, the following was prepared:

(A): *E. coli* DNA, 5 ng (1  $\mu$ l); Pst390, 100 pmoles (1  $\mu$ l);  
Pst391, 100 pmoles (1  $\mu$ l); dNTPs, 20 mM (1  $\mu$ l); 10xVent  
15 buffer (8  $\mu$ l); H<sub>2</sub>O, 68  $\mu$ l

(B): 10xVent buffer (3  $\mu$ l), Vent-polymerase, 3  $\mu$ l of 2 units/ $\mu$ l, H<sub>2</sub>O, 24  $\mu$ l.

A 40  $\mu$ l quantity of (A) was heated to 95°C after which 10  $\mu$ l of (B), also heated to 95°C, was added; the  
20 resultant mixture was subjected to 25 rounds of temperature cycling: 1 cycle = 1 minute at 95°C (denature), 1 minute at 55°C (anneal) and 1.5 minutes at 72°C (elongate). A second mixture of (A) and (B) was subjected to 35 temperature cycles. One-tenth of both PCR reactions was checked on  
25 agarose gel: each one the two amplifications gave a prominent band of the expected size (about 550 bp) following ethidium bromide staining. The PCR product resulting from the 35-cycle amplification was isolated from gel and subsequently digested with the enzymes BamHI and  
30 HindIII.

The recipient pMa5-19 plasmid (see Fig. 1A) was opened with BamHI and HindIII digestion. The large vector-fragment was purified from agarose gel and ligated with the

above-mentioned BamHI/HindIII digested PCR-product. The lacI<sup>q</sup> strain WK6 was transformed with the ligation mixture. Twelve randomly picked Ap<sup>R</sup> transformants were characterized by restriction analysis; one clone, designated pMa5-CI, was  
5 retained (Fig. 2).

The sequence of the entire Ecotin coding region of this clone was found to be in complete agreement with literature publications. Upon derepression of the P<sub>lac</sub> promoter, WK6 cells, harboring pMa5-CI, were found to  
10 direct the synthesis of Ecotin as shown by the dramatic increase in trypsin/factor Xa/elastase inhibitory activity. This activity can be released with an osmotic shock, demonstrating that Ecotin accumulates in the periplasmic space. Production of Ecotin could also be demonstrated by  
15 gel-electrophoretic fractionation of total cellular extracts followed by coomassie-staining; the protein profile of an IPTG-induced culture was found to contain a new protein with the expected molecular size. From the activity measurements it can be calculated that the Ecotin  
20 protein amounts to about 50 mg per liter culture (OD<sub>600 nm</sub> = ±4). Following purification (see below), the recombinant Ecotin was subjected to N-terminal sequencing. The result is consistent with literature data (Ala-Glu-Ser-Val-Gln-).

#### Example 7: Purification of Recombinant Ecotin

25 The overexpressed protein was purified from the periplasmic fraction. Cells from 2 L culture were resuspended in 120 ml 10 mM Tris pH 7.5, 20% sucrose, 10 mM EDTA and left for 10 minutes at room temperature before centrifugation. The pellet was subsequently resuspended in  
30 120 ml water. After 10 minutes the supernatant was collected by centrifugation and adjusted to pH 3.4 by the addition of HCl. Precipitated material was removed by

centrifugation. Solid NaCl to a final concentration of 0.3 M was added and the pH adjusted to 7.4. The liquid was subsequently heated for 30 minutes at 90°C.

Precipitated material was removed by  
5 centrifugation. Solid ammonium sulfate (430 g/L) was added and the suspension stored overnight at 4°C. After centrifugation, the pellet was dissolved in water, and the solution fractionated on Superdex 75 (16/60) gel filtration  
10 column. The fractions containing Ecotin were pooled and dialyzed extensively against water. Protein concentration was estimated using the calculated extinction coefficient,  $\epsilon_{278} = 1.47$  for 1.0 mg/ml. The molecular mass is 16,098 Daltons.

Example 8: Protease Assays

15 Protease activity was determined using commercially available chromogenic substrates and purified proteases. The initial rate was determined with a Thermomax (Molecular Devices) microtiterplate reader at 405 nm wavelength at room temperature. The buffer used for  
20 all measurements contained 100 mM TRIS, 140 mM NaCl, 0.1% BSA, pH 7.4.



The following protease assays were used:

	assayed protease	protease concentra- tion in assay (nM)	substrate	substrate concentra- tion in assay (mM)
	factor Xa	0.68	N- $\alpha$ -Cbo-D-Arg-Gly-Arg-pNa.2 HCl	0.25
5	trypsin	0.6	N-Bz-Ile-Glu-( $\gamma$ -OR)-Gly-Arg -pNA.HCl R=H (50%), CH <sub>3</sub> (50%)	0.25
	thrombin	2	H-D-Phe-Pip-Arg-pNA.2HCl	0.5
	p l a s m a kallikrein	0.025 nKat/ml	H-D-Pro-Phe-Arg-pNA.2HCl	0.4
10	f a c t o r VIIa-tissue factor	2.5	H-D-Ile-Pro-Arg-pNA.2HCl	0.4
	plasmin	1	L-pyroglutamyl-Pro-Arg-pNA. HCl	0.2
	elastase	10	N-succinyl-Ala-Ala-Ala-pNA	0.5
15	f a c t o r XIa	0.5	H-D-Val-Leu-Arg-pNA	0.25
	f a c t o r XIIa	1	D-hexahydroTyrosine-Gly-Arg -pNA diacetate	0.2
	aPC	1	$\gamma$ -Cbo-D-Lys-Pro-Arg-pNA diacetate	0.4
	rt-PA	1	O-(methylsulfonyl)-D-hexahy droTyrosine-Gly-Arg-pNA acetate	1
20	urokinase	1	O-(methylsulfonyl)-D-hexahy droTyrosine-Gly-Arg-pNA acetate	1

pNA=para-nitrophenylanilide,      Cbo=Benzoyloxycarbonyl,  
Bz=Benzoyl.

To determine inhibition constants, the protease was incubated during 90 minutes (factor Xa), 45 minutes (trypsin), or 30 minutes (thrombin, plasmin, factor VIIa-tissue factor, human plasma kallikrein, porcine pancreatic elastase, factor XIa, factor XIIa, activated protein C, recombinant t-PA and urokinase) in 150  $\mu$ l before the substrate (50  $\mu$ l) was added. The inhibition constant was determined from a plot of the relative activity versus the inhibitor concentration fitted with the following equation:

$$v_i/v_o = \{([E_t] - [I_t] - K_i^*) + \{([I_t] + K_i^* - [E_t])^2 + 4K_i^*[E_t]\}^{1/2}\} / 2[E_t]$$

except for thrombin where the total enzyme concentration can be neglected compared to the inhibitor concentration and the equation is reduced to:

$$v_i/v_o = 1 / (1 + [I_t]/K_i^*)$$

$v_i$  and  $v_o$  are the initial rates measured in presence and in absence of inhibitor,  $[I_t]$  and  $[E_t]$  are the total inhibitor and enzyme concentrations and  $K_i^*$  is the apparent inhibition constant. It was assumed that under these conditions the apparent inhibition constant is not dependent on the substrate concentration and corresponds to the binding constant of the inhibitor to the active site of the protease in the conditions of the experiment.

The factor VIIa-tissue factor complex was not inhibited by recombinant Ecotin at concentrations up to 1  $\mu$ M. Plasmin inhibition by 1  $\mu$ M recombinant Ecotin was 15%. The

inhibition constants of recombinant Ecotin for various other proteases are given below.

Protease	Inhibition constant (nM)
factor Xa	0.05 - 0.2
5 trypsin	0.01
thrombin	7200
human plasma kallikrein	1.4
factor XIa	2.5 - 25
10 factor XIIa	<<<2.5
chymotrypsin	<<<2.5
plasmin	inactive
activated protein C	>>2500
rt-PA	>>2500
15 urokinase	>>2500

Example 9: Rat Model of FeCl<sub>3</sub>-induced Platelet-dependent Arterial Thrombosis

The rat model is a well characterized model of platelet dependent, arterial thrombosis which has been used in the evaluation potential antithrombotic compounds such as direct thrombin inhibitors. Kurz et al., 60 Thromb. Res. 269, 1990. Thrombus development in this model is relatively heparin insensitive, which suggests that this model may be more representative of the type of thrombosis which has been observed clinically in newly re-canalized coronary vessels following balloon angioplasty or enzymatic thrombolysis. In

this model a platelet-rich, occlusive thrombus is formed in a segment of the rat carotid artery treated with a fresh solution of FeCl<sub>3</sub> absorbed to a piece of filter paper. The FeCl<sub>3</sub> is thought to diffuse into the treated segment of artery and causes de-endothelialization resulting in thrombus formation. The effect of a test compound on the incidence of occlusive thrombus formation following the application of the FeCl<sub>3</sub> is monitored by ultrasonic flowtometry, and is used as the primary end point. The use of flowtometry is a modification of the original procedure in which thermal detection of clot formation was employed. Kurz et al., supra.

Male Harlan Sprague Dawley rats (420-450 g) were acclimated at least 72 hours prior to use and fasted for 12 hours prior to surgery with free access to water. The animals were prepared, anesthetized with Nembutal with catheters for blood pressure monitoring, drug and anesthesia delivery being implanted as described above. The left carotid artery was isolated by making a midline cervical incision followed by blunt dissection and spreading techniques to separate a 2 cm segment of the vessel from the carotid sheath. A silk suture was inserted under the proximal and distal ends of the isolated vessel to provide clearance for the placement of an ultrasonic flow probe (Transonic) around the proximal end of the vessel. The probe was then secured with a stationary arm.

Following surgery, the animals were randomized in either a control (saline infusion) or treatment group with test compound (Ecotin) with at least 4 animals per group at the indicated dose. The test compound was administered as a continuous intravenous infusion after placement of the flow probe and stabilization of the preparation for a period of 60 minutes prior to and following application of the FeCl<sub>3</sub>. At

t=0, a 3 mm diameter piece of filter paper (Whatman #3) soaked with 10  $\mu$ l of a 35% solution of fresh FeCl<sub>3</sub> (made up in water) was applied to the segment of isolated carotid artery distal to the flow probe. Blood pressure, blood flow, heart rate, and respiration were monitored for 60 minutes.

The incidence of occlusion (defined as the attainment of zero blood flow) was recorded as the primary end point. Following the 60 minute observation period, the flow probe was removed and the area cleared of all excess fluid. The distal and proximal sutures were tied off and arterial clamps placed on the far proximal and distal ends of the segment. The isolated segment was cut out, blotted dry on filter paper and weighed. The segment was re-weighed following removal of the clot and the difference recorded as total % clot weight. The animals were euthanized as described above.

The efficacy of Ecotin, produced as described above, as an antithrombotic agent in this *in vivo* model was demonstrated by the reduction in the incidence of occlusion and in clot size, as shown below.

Treatment Group	Incidence of Occlusion	Time to Occlusion (min)	Clot Formation (%)
25 Control (No Treatment)	4/4	16.8 $\pm$ 1.3	60.83 $\pm$ 5.56
Ecotin 10 $\mu$ g/kg/min	2/6	20.1 $\pm$ 0.9	12.73 $\pm$ 8.04*
30 * p<0.01 vs Control by ANOVA followed by Newman-Kuels test			

Other embodiments are within the following claims.

Claims

1. Pharmaceutical composition adapted for inhibiting blood coagulation comprising an effective amount of Ecotin.
- 5           2. Method for inhibiting factor Xa activity, comprising contacting factor Xa with Ecotin.
3. Method for inhibiting factor XIa activity, comprising contacting factor XIa with Ecotin
4. Method for inhibiting factor XIIa activity,  
10 comprising contacting factor XIIa with Ecotin

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FIG. 1A

(A)

GAATTCGAGC TCGAGCTTAC TCCCATCC CCTGTGACA ATTAATCATC GGCTCGTATA  
 EcoRI -10

ATGTCTGGAA TTGTGACGG ATAACAATT CACACAGGAA ACAGGATCCG CGGATCCGTG  
 BamHI BamHI

GAGAAAATAA A ATG AAA CAA AGC ACT ATT GCA CTG GCA CTC TTA CCG TTA  
 SD Met Lys Gln Ser Thr Ile Ala Leu Ala Leu Leu Pro Leu

CTG TTT ACC CCT GTG ACA AAA GCG GTACCCGGG ATCCTCTAGA GTCGACCTGC  
 Leu Phe Thr Pro Val Thr Lys Ala KpnI

AGGCATGCAA GCTTGGTCTA GA  
 HindIII XbaI

(B)

10	20	30	40	50
*	*	*	*	*
GC GAC ATC ATC GGT GGG ATC CTA TAA ATG AAG AAG GAA AGC AAA AAA ATG AAG ACC ATT				
	BamHI			Met Lys Thr Ile
60	70	80	90	100
*	*	*	*	*
PstI				
CTA CCT GCA GTA TTG TTT GCC GCT TTC GCT ACC ACT TCC GCC TGG GCG GCA AGC GTC				
Leu Pro Ala Val Leu Phe Ala Ala Phe Ala Thr Thr Ser Ala Trp Ala Ala Glu Ser Val				
120	130	140	150	160
*	*	*	*	*
CAG CCA CTG GAA AAA ATC GCG CCT TAT CCA CAA GCT GAA AAA GGG ATG AAG CGT CAG GTG				
Gln Pro Leu Glu Lys Ile Ala Pro Tyr Pro Gln Ala Glu Lys Gly Met Lys Arg Gln Val				
180	190	200	210	220
*	*	*	*	*
ATT CAG TTA ACC CCG CAA GAA GAT GAA TCT ACC CTG AAA GTA GAA CTG TTA ATC GGT CAG				
Ile Gln Leu Thr Pro Gln Glu Asp Glu Ser Thr Leu Lys Val Glu Leu Ile Gly Gln				

25

240 *	250 *	260 *	270 *	280 *	290 *
ACG CTG GAA GTC GAT TGC AAT TTG CAT CGT CTC GGC GGG AAG CTG GAA AAC AAA ACG CTG					
Thr Leu Glu Val Asp Cys Asn Leu His Arg Leu Gly Gly Lys Leu Glu Asn Lys Thr Leu					
300 *	310 *	320 *	330 *	340 *	350 *
GAA GGC TGG GGC TAT GAT TAT GTC TTT GAT AAA GTC AGT TCC CCG GTT TCA ACG ATG					
Glu Gly Trp Gly Tyr Asp Tyr Tyr Val Phe Asp Lys Val Ser Ser Pro Val Ser Thr Met					
360 *	370 *	380 *	390 *	400 *	410 *
ATG GCC TGC CCG GAT GGC AAG AAA GAG AAG AAA TTT GTC ACC GCG TAT CTG GGC GAT GCT					
Met Ala Cys Pro Asp Gly Lys Lys Glu Lys Phe Val Thr Ala Tyr Leu Gly Asp Ala					
420 *	430 *	440 *	450 *	460 *	470 *
GGA ATG CTG CGT TAC AAC AGC AAG CTG CCG ATC GTG GTG TAT ACG CCA GAC AAT GTA GAT					
Gly Met Leu Arg Tyr Asn Ser Lys Lys Leu Pro Ile Val Val Tyr Thr Pro Asp Asn Val Asp					
480 *	490 *	500 *	510 *	520 *	530 *
GTG AAG TAC CGC GTC TGG AAG GCG GAA GAG AAA ATT GAC AAC ACG GCG GTA GTT CGC TAA GCT					
Val Lys Tyr Arg Val Trp Lys Ala Glu Glu Lys Ile Asp Asn Ala Val Val Arg ***					
540 *	550 *				
TCC GTG AAG TGC					

FIG. 1B



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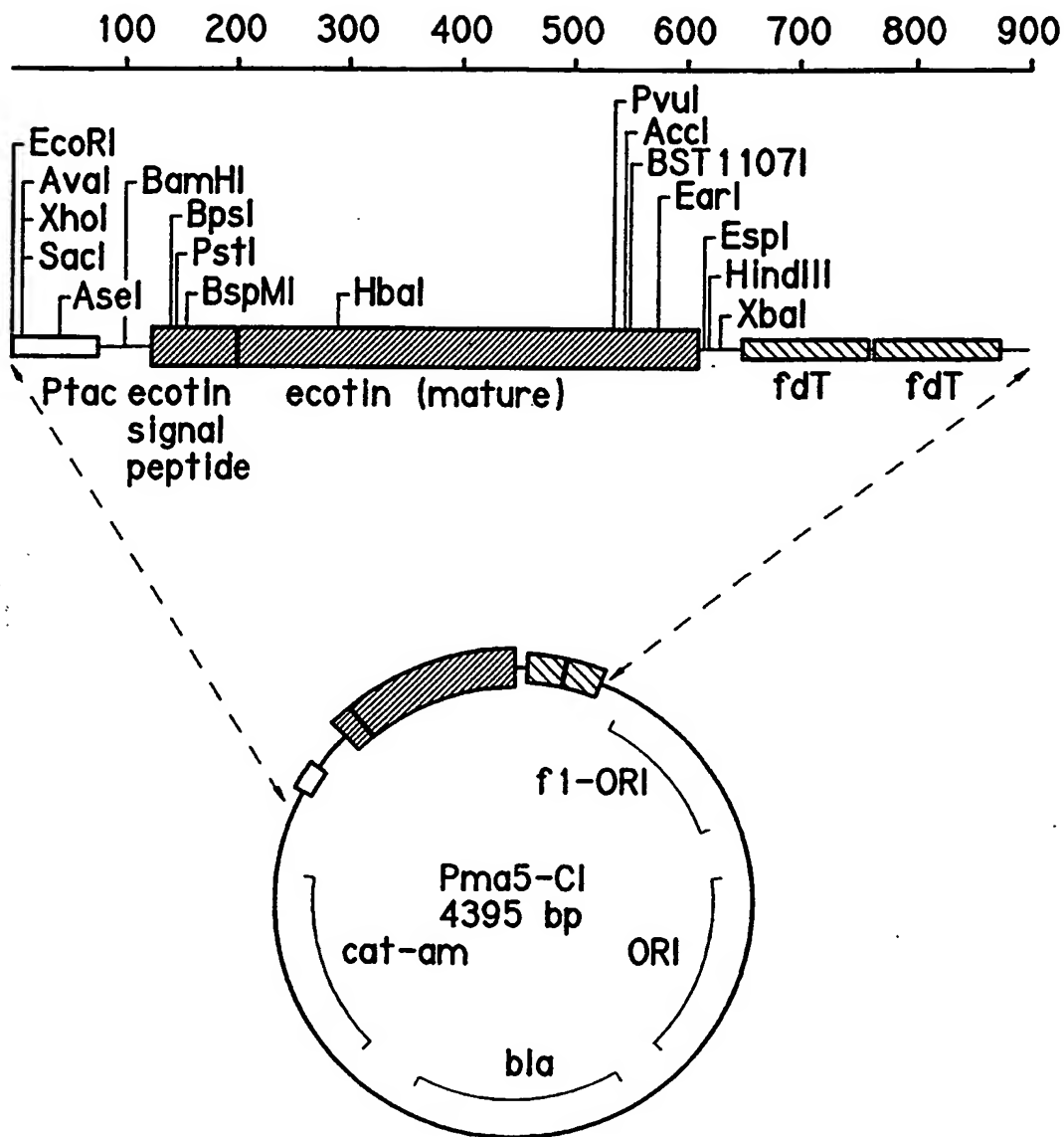
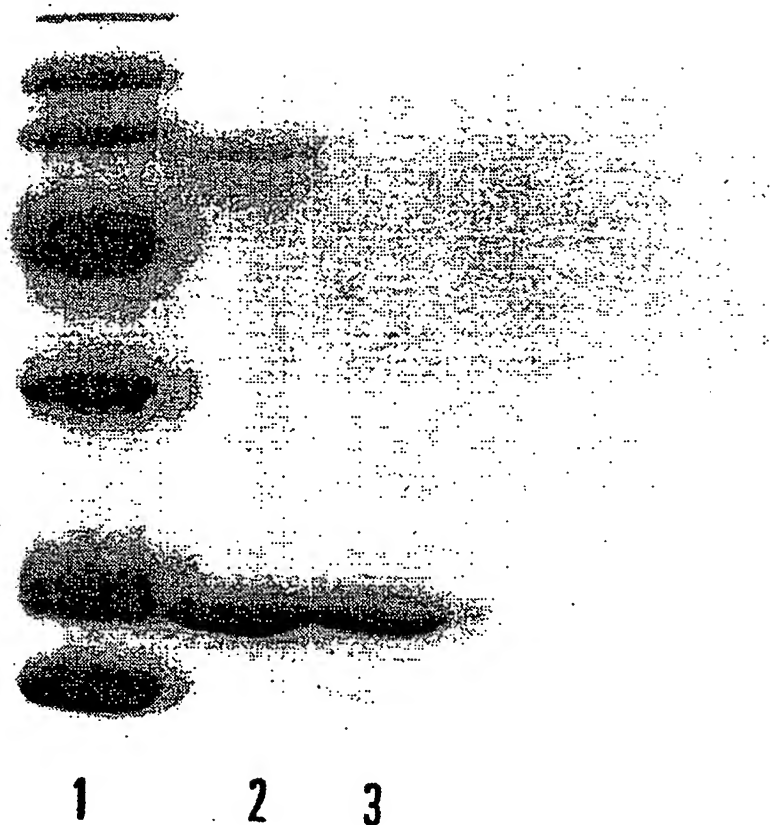


FIG. 2

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FIG. 3.



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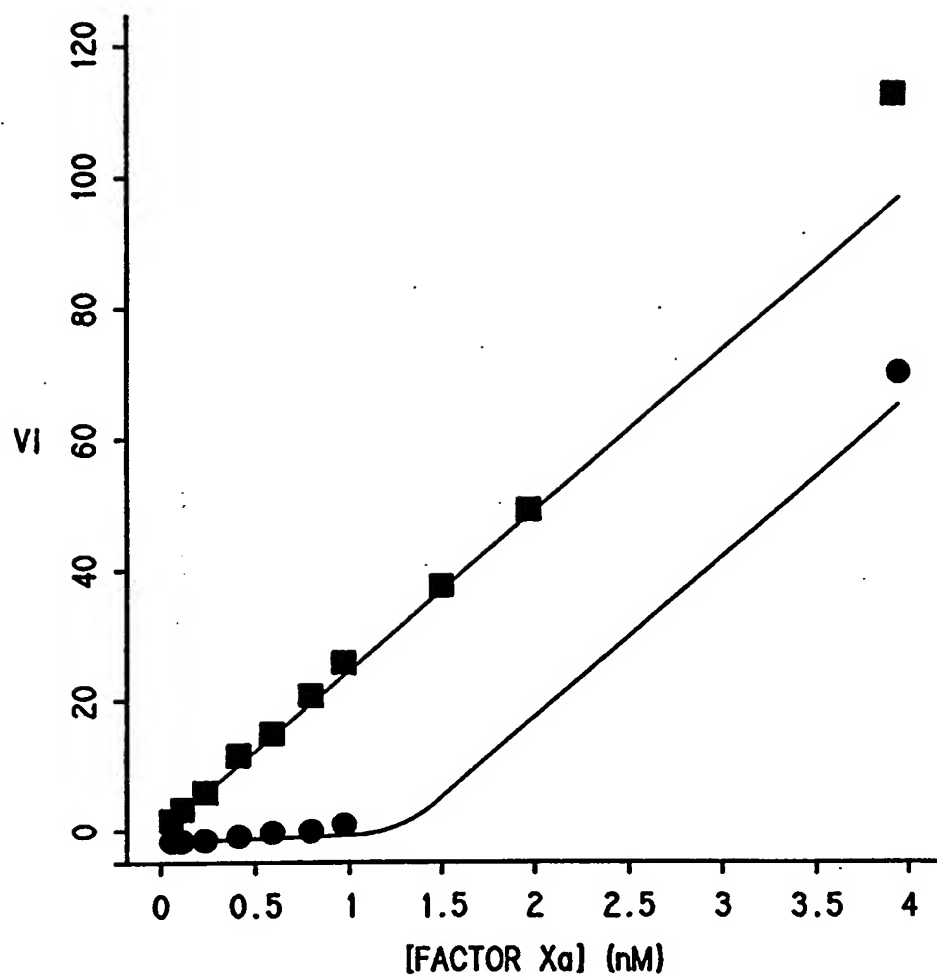


FIG. 4

## INTERNATIONAL SEARCH REPORT

I. International application No.

PCT/US93/12003

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) : C07K 13/00; C12N 9/48, 15/31; A61K 35/00, 35/74

US CL : 424/94.64; 435/212, 226, 317.1; 514/12; 530/350, 825; 930/250

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.64; 435/212, 226, 317.1; 514/12; 530/350, 825; 930/250

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

ENZYME HANDBOOK, Schomburg &amp; Salzmann, eds.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, MEDLINE, EMBASE

search terms: ecotin, serine protease, factor X, factor XI, factor XII

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Journal of Molecular Biology, Volume 222, issued 1991, M.E. McGrath et al, "Expression of the Protease Inhibitor Ecotin and Its Cocrystallization with Trypsin", pages 139-142, see entire document.	1
X	Journal of Biological Chemistry, Volume 258, No. 18, issued 25 September 1982, C.H. Chung et al, "Purification from <i>Escherichia coli</i> of a Periplasmic Protein That Is a Potent Inhibitor of Pancreatic Proteases", pages 11032-11038, see entire document.	1
Y	D. Schomburg et al., "Enzyme Handbook", published by Springer-Verlag (Germany), see section 3.4.21, especially subsections 3.4.21.4, 3.4.21.6, 3.4.21.27, 3.4.21.38.	2-4

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Z" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 15 March 1994	Date of mailing of the international search report MAR 25 1994
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